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PATENT
2815-127P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: Thomas J. JENTSCH

Appl. No.: 09/492,361 Group:

Filed: January 27, 2000 Examiner:

For: NOVEL POTASSIUM CHANNELS AND GENES ENCODING THESE POTASSIUM CHANNELS

LETTER

Assistant Commissioner for Patents
Washington, DC 20231

April 10, 2000

Sir:

Under the provisions of 35 U.S.C. § 119 and 37 C.F.R. § 1.55(a), the applicant(s) hereby claim(s) the right of priority based on the following application(s):

Country	Application No.	Filed
DENMARK	1999 00076	January 26, 1999
DENMARK	1999 00693	May 19, 1999

A certified copy of the above-noted application(s) is(are) attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fee required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By ______________________
Gerald M. Murphy, Jr., #28,977

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

GMM/MAA:jls
2815-127P

Attachment

OIPE JC35
APR 1 0 2000
PATENT & TRADEMARK OFFICE

Kongeriget Danmark

Patent application No.: PA 1999 00076

Date of filing: 26 Jan 1999

Applicant: NeuroSearch A/S
Pederstrupvej 93
DK-2750 Ballerup

This is to certify the correctness of the following information:

The attached photocopy is a true copy of the following document:

- The specification, claims, abstract and drawings abstract as filed with the application on the filing date indicated above

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TAASTRUP 20 Jan 2000

Lizzi Vester

Lizzi Vester
Head of Section

Modtaget PD

26 JAN. 1999

NOVEL POTASSIUM CHANNELS AND GENES ENCODING THESE POTASSIUM CHANNELS

TECHNICAL FIELD

This invention relates to novel potassium channels and genes encoding these channels. More specifically the invention provides isolated polynucleotides encoding the KCNQ4 potassium channel subunit, cells transformed with these polynucleotides, transgenic animals comprising genetic mutations, and the use of the transformed cells and the transgenic animals for the *in vitro* and *in vivo* screening of chemical compounds affecting KCNQ4 subunit containing potassium channels.

BACKGROUND ART

Potassium channels participate in the regulation of electrical signalling in excitable cells, and regulates the ionic composition of biological fluids. Mutations in the three known genes of the *KCNQ* branch of the K^+-channel gene family underlie inherited cardiac arrhythmias, in some cases associated with deafness, and neonatal epilepsy.

Hearing loss is the most frequent sensory defect in humans. Hearing loss can be due to environmental and genetic factors, and the progressive hearing loss of the elderly (presbyacusis) most often seems to be due to a combination of both.

Inherited deafness can be classified as non-syndromic (isolated hearing loss) or syndromic (associated with other anomalies). Several hundred syndromes, consisting of hearing loss associated with defects in a variety of other organ systems, have been described. Nonsyndromic deafness is classified according to its mode of inheritance as DFN, DFNA, and DFNB (X-linked, autosomal dominant and autosomal recessive, respectively). In general, autosomal recessive deafness has an early onset and is very severe. Autosomal dominant deafness, by contrast, more often develops slowly over several decades and may become apparent only in adulthood. It is hoped that genes identified in families with dominant deafness may also - with different types of mutations - underlie some forms of presbyacusis.

A bewildering number of loci for non-syndromic deafness were identified in the last four years. There are at least 19 loci for autosomal dominant deafness (DFNA1 to DFNA19), and 22 loci for DFNB. Sometimes, depending on the particular mutation, the same gene can be involved in dominant or recessive deafness. This large number of loci reflects the complexity of the inner ear. Identification of these genes and characterisation of their products will significantly advance our understanding of the molecular basis of the physiology of this sensory organ.

Several genes involved in syndromic and non-syndromic deafness have already been identified and are reviewed by *Petit* [*Petit C*: Genes responsible for human hereditary deafness: symphony of a thousand; <u>Nature Genet.</u> 1996 **14** 385-391] and *Kalatzis & Petit* [*Kalatzis V & Petit C*: The fundamental and medical impacts of recent progress in research on hereditary hearing loss; <u>Hum. Mol. Genet.</u> 1998 **7** 1589-1597]. Among others, their gene products include transcription factors, unconventional myosin isoforms, α-tectorin (an extracellular matrix protein), diaphanous, a protein interacting with the cytoskeleton, connexin 26 (a gap junction protein), and two genes encoding potassium channel subunits, *KCNQ1* and *KCNE1*.

Ion channels play important roles in signal transduction and in the regulation of the ionic composition of intra- and extracellular fluids. Mutations in ion channels were since long suspected as possibly underlying some forms of hearing loss. In the cochlea (the auditory sensory organ), the transduction current through the sensory cells is carried by potassium ions and depends on the high concentration of that ion in the endolymph. So far only two genes encoding potassium channel subunits, *KCNQ1* and *KCNE1*, were found to be mutated in syndromic hereditary deafness. The gene products of both genes, the KCNQ1 (or KvLQT1) and the minK (or IsK) protein, respectively, form heteromeric potassium channels.

KCNQ1 is a typical member of the voltage-gated potassium channel superfamily with 6 transmembrane domains and a pore region situated between the fifth and the sixth transmembrane domain. The minK protein has a single transmembrane span and cannot form potassium channels on its own. However, as a ß-subunit it enhances and modifies currents mediated by KCNQ1. These heteromeric channels participate in the repolarisation of the heart action potential. Certain mutations in either *KCNQ1* or *KCNE1* cause a form of the autosomal dominant long QT syndrome (LQTS), a disease characterised by repolarisation anomalies of cardiac

action potentials resulting in arrhythmias and sudden death. Interestingly, other mutations in either gene lead to the recessive Jervell and Lange-Nielsen (JLN) syndrome that combines LQTS with congenital deafness. In order to cause deafness, KCNQ1/minK currents must be reduced below levels that are already sufficiently low to cause cardiac arrhythmia.

SUMMARY OF THE INVENTION

We have now cloned and characterised KCNQ4, a novel member of the KCNQ family of potassium channel proteins. *KCNQ4* has been mapped to the DFNA2 locus for autosomal dominant hearing loss, and a dominant negative *KCNQ4* mutation that causes deafness in a DFNA2 pedigree was identified.

KCNQ4 is the first potassium channel gene underlying non-syndromic deafness. KCNQ4 forms heteromeric channels with other KCNQ channel subunits, in particular KCNQ3.

The present invention has important implications for the characterisation and exploitation of this interesting branch of the potassium channel super family, as well as for the understanding of the cochlear physiology, and for human deafness and progressive hearing loss.

Accordingly, in its first aspect, the invention provides an isolated polynucleotide having a nucleic acid sequence which is capable of hybridising under high stringency conditions with the polynucleotide sequence presented as SEQ ID NO: 1, its complementary strand, or a sub-sequence thereof.

In another aspect the invention provides a recombinantly produced polypeptide encoded by the polynucleotide of the invention.

In a third aspect the invention provides a cell genetically manipulated by the incorporation of a heterologous polynucleotide of the invention.

In a fourth aspect the invention provides a method of screening a chemical compound for inhibiting or activating or otherwise modulating the activity on a potassium channel comprising at least one KCNQ4 channel subunit, which method comprises the steps of subjecting a KCNQ4 channel subunit containing cell to the action of the chemical compound; and monitoring the membrane potential, the current,

the potassium flux, or the secondary calcium influx of the KCNQ4 channel subunit containing cell.

In a fifth aspect the invention relates to the use of a polynucleotide sequence of the invention for the screening of genetic materials from humans suffering from loss of hearing (e.g. dominant, recessive, or otherwise), tinnitus, and other neurological diseases for mutations in the *KCNQ4* gene.

In a sixth aspect the invention relates to the chemical compound identified by the method of the invention, in particular to the use of such compounds for diagnosis, treatment or alleviation of a disease related to tinnitus; loss of hearing, in particular progressive hearing loss, neonatal deafness, and presbyacusis (deafness of the elderly); and diseases or adverse conditions of the CNS, including affective disorders, Alzheimer's disease, anxiety, ataxia, CNS damage caused by trauma, stroke or neurodegenerative illness, cognitive deficits, compulsive behaviour, dementia, depression, Huntington's disease, mania, memory impairment, memory disorders, memory dysfunction, motion disorders, motor disorders, neurodegenerative diseases, Parkinson's disease and Parkinson-like motor disorders, phobias, Pick's disease, psychosis, schizophrenia, spinal cord damage, stroke, and tremor.

In a seventh aspect the invention provides a transgenic animal comprising a knock-out mutation of the endogenous *KCNQ4* gene, a replacement by or an additional expression of a mutated *KCNQ4* gene, or genetically manipulated in order to over-express the *KCNQ4* gene or to over-express mutated *KCNQ4* gene.

In an eighth aspect the invention relates to the use of the transgenic animal of the invention for the *in vivo* screening of therapeutic compounds.

Other objects of the invention will be apparent to the person skilled in the art from the following detailed description and examples.

DETAILED DISCLOSURE OF THE INVENTION

The present invention provides novel potassium channels and genes encoding these channels. The invention also provides cells transformed with these genes, transgenic animals comprising genetic mutations, and the use of the transformed cells and the transgenic animals for the *in vitro* and *in vivo* screening of drugs affecting KCNQ4 containing potassium channels.

Polynucleotides

In its first aspect, the invention provides novel polynucleotides.

The polynucleotides of the invention are such which have a nucleic acid sequence capable of hybridising under high stringency conditions with the polynucleotide sequence presented as SEQ ID NO: 1, its complementary strand, or a sub-sequence thereof.

Hybridisation Protocol

The polynucleotides of the invention are such which have a nucleic acid sequence capable of hybridising with the polynucleotide sequence presented as SEQ ID NO: 1, its complementary strand, or a sub-sequence thereof, under at least medium, medium/high, or high stringency conditions, as described in more detail below.

Suitable experimental conditions for determining hybridisation at medium/high or high stringency conditions between a nucleotide probe and a homologous DNA or RNA sequence, involves pre-soaking of the filter containing the DNA fragments or RNA to hybridise in 5 x SSC [Sodium chloride/Sodium citrate; cf. *Sambrook et al.*; Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Lab., Cold Spring Harbor, NY 1989] for 10 minutes, and pre-hybridisation of the filter in a solution of 5 x SSC, 5 x Denhardt's solution [cf. *Sambrook et al.; Op cit.*], 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA [cf. *Sambrook et al.; Op cit.*], followed by hybridisation in the same solution containing a concentration of 10 ng/ml of a random-primed [*Feinberg A P & Vogelstein B*; Anal. Biochem. 1983 **132** 6-13], ^{32}P-dCTP-labeled (specific activity > 1×10^9 cpm/µg) probe for 12 hours at approximately 45°C. The filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS at a temperature of at least at least 60°C (medium stringency conditions), preferably of at least 65°C (medium/high stringency conditions), more preferred of at least 70°C (high stringency conditions), and even more preferred of at least 75°C (very high stringency conditions).

Molecules to which the oligonucleotide probe hybridises under these conditions may be detected using a x-ray film.

DNA Sequence Homology

In a preferred embodiment, the polynucleotides of the invention show a homology of at least 50%, preferably at least 70%, more preferred at least 80%, even more preferred at least 90%, most preferred at least 95%, with the polynucleotide sequence presented as SEQ ID NO: 1.

As defined herein, the DNA sequence homology may be determined as the degree of identity between two DNA sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package [*Needleman S B and Wunsch C D*, Journal of Molecular Biology 1970 **48** 443-453] using default parameters suggested herein.

Cloned Polynucleotides

The isolated polynucleotide of the invention may in particular be a cloned polynucleotide.

As defined herein, the term "cloned polynucleotide", refers to a polynucleotide or DNA sequence cloned in accordance with standard cloning procedures currently used in genetic engineering to relocate a segment of DNA, which may in particular be cDNA, i.e. enzymatically derived from RNA, from its natural location to a different site where it will be reproduced.

Cloning may be accomplished by excision and isolation of the desired DNA segment, insertion of the piece of DNA into the vector molecule and incorporation of the recombinant vector into a cell where multiple copies or clones of the DNA segment will be replicated, by reverse transcription of mRNA (reverse transcriptase technology), and by use of sequence-specific oligonucleotides and DNA polymerase in a polymerase chain reaction (PCR technology).

The cloned polynucleotide of the invention may alternatively be termed "DNA construct" or "isolated DNA sequence", and may in particular be a complementary DNA (cDNA).

It is well established that potassium channels may be formed as heteromeric channels, composed of different subunits. Also it has been found that the potassium channel of the invention may form heteromers with other KCNQ's, in particular KCNQ3, when co-expressed with these subunits. In addition, potassium

channels can also associate with non-homologous subunits, e.g. the KCNE1 (formerly known as minK) subunit, that can functionally modulate these channels or lead to a specific localisation within the cell.

Therefore, in a preferred embodiment, the polynucleotide of the invention is cloned and either expressed by itself or co-expressed with polynucleotides encoding other subunits, in particular a polynucleotide encoding a KCNQ3 channel subunit.

Biological Sources

The isolated polynucleotide of the invention may be obtained from any suitable source. In a preferred embodiment, which the polynucleotide of the invention is cloned from, or produced on the basis of a cDNA library, e.g. of the retina, brain, skeletal muscle. Commercial cDNA libraries are available from e.g. Stratagene and Clontech.

The isolated polynucleotide of the invention may be obtained methods known in the art, e.g. those described in the working examples below.

Preferred Polynucleotides

In a preferred embodiment, polynucleotide of the invention has the polynucleotide sequence presented as SEQ ID NO: 1.

In another preferred embodiment the polynucleotide of the invention is a sequence giving rise to KCNQ4 channels subunits comprising one or more substitutions.

In another preferred embodiment the polynucleotide of the invention is a sequence giving rise to KCNQ4 channels subunits comprising one or more substitutions in the conserved regions, as defined in more details below.

In a more preferred embodiment the polynucleotide of the invention has the polynucleotide sequence giving rise to the G285S mutation as indicated in SEQ ID NO: 1, i.e. the DNA sequence that at position 935-937 holds the codon AGC rather than the codon GGC stated in SEQ ID NO: 1.

Also contemplated within the scope of this invention are the primer sequences used in Example 2 below for the amplification of the single KCNQ4 exons, that can then be screened for mutations.

It has been demonstrated that KCNQ channels often show alternative splicing and therefore may occur as isoforms originating from the same gene. Such

isoforms as well as the different cDNA sequences from which they occurred are also contemplated within the scope of the present invention.

Finally the genes encoding KCNQ channel subunits in other species have been found to differ slightly from the human genes. However, genes of other species, e.g. mouse, rat, monkey, rabbit, etc., are also contemplated within the scope of the present invention.

Recombinantly Produced Polypeptides

In another aspect the invention relates to, and provides, the novel polypeptides that may be obtained by the polynucleotides of the invention using standard recombinant DNA technology known in the art.

In a preferred embodiment, a polypeptide of the invention is the KCNQ4 potassium channel subunit having the amino acid sequence presented as SEQ ID NO: 2. However, variants of this protein are also contemplated according to the invention, including splice variants, isoforms, homologues from other species, and polymorphisms, or mutations including the variant KCNQ4/G285S, as described in more detail below.

Also contemplated within the scope of this invention are the oligonucleotides encoded by the primer sequences used in Example 2 below for the amplification of the single KCNQ4 exons, that can then be screened for mutations.

<u>KCNQ1 Numbering System</u>

In the context of this invention, amino acid residues (as well as nucleic acid bases) are specified using the established one-letter symbol.

By aligning the amino acid sequences of a polypeptide of the present invention to those of the known polypeptides, a specific amino acid numbering system may be employed, by which system it is possible to unambiguously allot an amino acid position number to any amino acid residue in any KNCQ channel protein, which amino acid sequence is known.

Such an alignment is presented in Table 1, below. Using the ClustalX computer alignment program [*Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, & Higgins DG*: The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools; <u>Nucleic Acids Res.</u> 1997 **25** (24) 4876-82],

and the default parameters suggested herein, the amino acid sequence of a polypeptide of the present invention (hKCNQ4) and the amino acid sequences of the known polypeptides hKCNQ2-3 are aligned with, and relative to, the amino acid sequences of the known polypeptide hKCNQ1 (formerly known as KvLQT1). In the context of this invention this numbering system is designated the KCNQ1 Numbering System.

In describing the various protein variants produced or contemplated according to the invention, the following nomenclatures have been adapted for ease of reference:

Original amino acid / Position / Substituted amino acid

According to this nomenclature the substitution of serine for glycine at position 333 is designated as "G333S".

A deletion of glycine at the same position is designated "G333*".

An insertion of an <u>additional</u> amino acid residue, in this example lysine, may be designated "G333GK" or "*334K" (assumed that no position exists for this position in the amino acid sequence used for establishing the numbering system).

An insertion of an amino acid residue, in this example valine, at a position which exists in the established numbering system, but where no amino acid residue is actually present, may be designated "-301V".

Table 1

CLUSTAL X Multiple Sequence Alignment

KCNQ1 Numbering

```
hKCNQ2   MVQKSR---- ---------- NGGVYPGPSG EKKLKVG--- -FVGLDPG-- -------APD  60
hKCNQ3   MGLKARRAAG AAGGGDGGG GGGGAANPAG GDAAAAGDEE RKVGLAPGDV EQVTLALGAG
hKCNQ4   MAEAPPR--- ---------- RLGLGPPPGD APRAELVALT -AVQSEQGE- -------AGG
hKCNQ1   MAAASSPPR- -AE--RKR-W GWGRLPGARR GSAGLAKKCP FSLELAEG-- -------GPA
         *    .                *  . .                 :     *          .

hKCNQ2   STRDGALLIA G--------S EAPKRGSILS KPRAGGAGAG KPPKRN-AFY RK-------L 120
hKCNQ3   ADKDGTLLLE GGG------R DEGQRRTPQG IGLLAKTPLS RPVKRNNAKY RR-------I
hKCNQ4   GGSPRRLGLL G--------S PLPPGAPLPG PGSGSGSACG QRSSAAHKRY RR-------L
hKCNQ1   GGALYAPIAP GAPGPAPPAS PAAPAAPPVA SDLGPRPPVS LDPRVSIYST RRPVLARTHV
         .          *                   .  .       .  .           *:        :

hKCNQ2   QNFLYNVLER PRGW-AFIYH AYVFLLVFSC LVLSVFSTIK EYEKSSEGAL YILEIVTIVV 180
hKCNQ3   QTLIYDALER PRGW-ALLYH ALVFLIVLGC LILAVLTTFK EYETVSGDWL LLLETFAIFI
hKCNQ4   QNWVYNVLER PRGW-AFVYH VFIFLLVFSC LVLSVLSTIQ EHQELANECL LILEFVMIVV
hKCNQ1   QGRVYNFLER PTGWKCFVYH FAVFLIVLVC LIFSVLSTIE QYAALATGTL FWMEIVLVVF
         *   :*: *** * ** .::**   :**:*: * *:::*::*:: ::    :    *   :* . :..

hKCNQ2   FGVEYFVRIW AAGCCCRYRG WRGRLKFARK PFCVIDIMVL IASIAVLAAG SQGNVFATSA 240
hKCNQ3   FGAEFALRIW AAGCCCRYKG WRGRLKFARK PLCMLDIFVL IASVPVVAVG NQGNVLATS-
hKCNQ4   FGLEYIVRVW SAGCCCRYRG WQGRFRFARK PFCVIDFIVF VASVAVIAAG TQGNIFATSA
hKCNQ1   FGTEYVVRLW SAGCRSKYVG LWGRLRFARK PISIIDLIVV VASMVVLCVG SKGQVFATSA
         ** *: :*:* :*** .:* *   **::**** *:.::*::*. :**: *:..* .:*:::***

hKCNQ2   LRSLRFLQIL RMIRMDRRGG TWKLLGSVVY AHSKELVTAW YIGFLCLILA SFLVYLAEK- 300
hKCNQ3   LRSLRFLQIL RMLRMDRRGG TWKLLGSAIC AHSKELITAW YIGFLTLILS SFLVYLVEKD
hKCNQ4   LRSMRFLQIL RMVRMDRRGG TWKLLGSVVY AHSKELITAW YIGFLVLIFA SFLVYLAEKD
hKCNQ1   IRGIRFLQIL RMLHVDRQGG TWRLLGSVVF IHRQELITTL YIGFLGLIFS SYFVYLAEKD
         :*.:****** **:::**:** **:****.:   * :**:*:  ***** **::  *::***.**

hKCNQ2   -------GE- -NDHFDTYAD ALWWGLITLT TIGYGDKYPQ TWNGRLLAAT FTLIGVSFFA 360
hKCNQ3   VPEVDAQGEE MKEEFETYAD ALWWGLITLA TIGYGDKTPK TWEGRLIAAT FSLIGVSFFA
hKCNQ4   ---------- ANSDFSSYAD SLWWGTITLT TIGYGDKTPH TWLGRVLAAG FALLGISFFA
hKCNQ1   -----AVNES GRVEFGSYAD ALWWGVVTVT TIGYGDKVPQ TWVGKTIASC FSVFAISFFA
                    . .* :*** :**** :*:: ******* *: ** *: :*:  *::::.:****
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hKCNQ4 is printed in bold throughout.

```
hKCNQ2  LPAGILGSGF ALKVQEQHRQ KHFEKRRNPA AGLIQSAWRF YATNLSRTDL HSTWQYYERT 420
hKCNQ3  LPAGILGSGL ALKVQEQHRQ KHFEKRRKPA AELIQAAWRY YATNPNRIDL VATWRFYESV
hKCNQ4  LPAGILGSGF ALKVQEQHRQ KHFEKRRMPA ANLIQAAWRL YSTDMSRAYL TATWYYYDSI
hKCNQ1  LPAGILGSGF ALKVQQKQRQ KHFNRQIPAA ASLIQTAWRC YAAE--NPD- SSTWKIYIRK
        *********: *****:::** ***:::  .* * ***:***  *:::  .      :**  *

hKCNQ2  VT-------- VPMYRLIPP- -LNQLELLRN LKSKSGLAFR K--------- DPPPEPSPSQ 480
hKCNQ3  VS-------- FPFFRKE--- ---QLEAAS- ---------- ---------- ------S--Q
hKCNQ4  LPSFRELALL FEHVQRARNG GLRPLEVRRA PVPDGAPSRY PPVATCHRPG STSFCPGESS
hKCNQ1  AP-------- RSHTLLS--- ---PSPKPKK ---------- ---------- ---------S
         .                                                           .

hKCNQ2  KVSLKDRV-F SSPRGVAAKG KGSPQAQTVR RSPSADQSLE D-SPSKVPKSW SFG-DRSRA 540
hKCNQ3  KLGLLDRVRL SNPRGSNTKG K--------L FTPLNVDAIE E-SPSKEPKPV GLN-NKERF
hKCNQ4  RMGIKDRIRM GSSQRRTGPS KQQLAPPTMP TSPSSEQVGE ATSPTKVQKSW SFN-DRTRF
hKCNQ1  VVVKKKKFKL DKDNGVTPGE K-MLTVPHIT CDPPEERRLD HFSVDGYDSSV RKSPTLLEV
          :   .:. : .. .        *            *        :    *      ..    .    .

hKCNQ2  RQAFRIKGAA SRQNSEEASL PGEDIVDDKS CPCEFVTEDL TPGLKVSIRA VCVMRFLVSK 600
hKCNQ3  RTAFRMKAYA FWQSSEDAGT -GDPMAEDRG YGNDFPIEDM IPTLKAAIRA VRILQFRLYK
hKCNQ4  RASLRLKP-- -RTSAEDAPS --EEVAEEKS YQCELTVDDI MPAVKTVIRS IRILKFLVAK
hKCNQ1  SMPHFMRT-- -NSFAEDLDL EGETLLTPIT H-----ISQL REHHRATIKV IRRMQYFVAK
          .  ::         :*:       : :                .::        :. *:  :  ::: : *

hKCNQ2  RKFKESLRPY DVMDVIEQYS AGHLDMLSRI KSLQSRVDQI VGRGP----A ITDKDR—TK  660
hKCNQ3  KKFKETLRPY DVKDVIEQYS AGHLDMLSRI KYLQTRIDMI FTPGP----P STPKHKKSQK
hKCNQ4  RKFKETLRPY DVKDVIEQYS AGHLDMLGRI KSLQTRVDQI VGRGP----G DRKAREKGDK
hKCNQ1  KKFQQARKPY DVRDVIEQYS QGHLNLMVRI KELQRRLDQS IGK-PSLFIS VSEKSK--DR
        :**::: :** ** *******  ***::: ** * ** *:*     .    *            .   :

hKCNQ2  G--------- ---------- PAEAELPEDP SMMGRLGKVE KQVLSMEKKL DFLVNIY--M 720
hKCNQ3  GSAFTFPSQQ SPRNEPYVAR PSTSEI-EDQ SMMGKFVKVE RQVQDMGKKL DFLVDMH--M
hKCNQ4  G--------- ---------- PSDAEVVDEI SMMGRVVKVE KQVQSIEHKL DLLLGFYSRC
hKCNQ1  G--------- ---------- --------SN TIGARLNRVE DKVTQLDQRL ALITDML---
        *                             .  :: .:. :**  :* .: ::*   :: .:

hKCNQ2  QRMGIPPTET EAYFGAKEPE PAPPYHSPED SREHVDRHGC IVKIVRSSSS TGQKNF---S 780
hKCNQ3  QHMERLQVQV TEYYPTKGTS SPAEAEKKED NR-YSDLKTI ICNYSETGPP EPPYSFHQVT
hKCNQ4  LRSGTSASLG AVQVPLFDPD ITSDYHSPVD HE-DISVSAQ TLSISRSVST NMD-------
hKCNQ1  -HQLLSLHGG STP-GSGGPP REGGAHITQP CGSGGSVDPE LFLPSNTLPT YEQLTV----
          :                .        .          .             .: ..

hKCNQ2  APPAAPPVQC PPSTSWQPQS HPRQG--HGT SPVGDHGSLV RIPPPPAHER SLSAYGGGNR 840
hKCNQ3  IDKVSPYGFF AHDPVNLPRG GPSSGKVQAT PPSSATTYVE RPTVLPILTL LDSRVSCHSQ
hKCNQ4  ---------- ---------- ---------- ---------- ---------- ----------
hKCNQ1  PRRGPDEGS- ---------- ---------- ---------- ---------- ----------
```

```
hKCNQ2   ASMEFLRQED TPGCRPPEGT LRDSDTSISI PSVDHEELER SFSGFSISQS KENLDALNSC 900
hKCNQ3   ADLQGP-YSD RISPRQRRSI TRDSDTPLSL MSVNHEELER SPSGFSISQD RD--DYVFGP
hKCNQ4   ---------- ---------- ---------- ---------- ---------- ----------
hKCNQ1   ---------- ---------- ---------- ---------- ---------- ----------

hKCNQ2   YAAVAPCAKV RPYIAEGESD TDSDLCTPCG PPPRSATGEG PFGDVGWAGP RK--       954
hKCNQ3   NGGSSWMREK R-YLAEGETD TDTDPFTPSG SMPLSSTGDG -ISDSVWTPS NKPI
hKCNQ4   ---------- ---------- ---------- ---------- ---------- ----
hKCNQ1   ---------- ---------- ---------- ---------- ---------- ----
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hKCNQ1: Human KCNQ1 [*Wang, Q et al.*, Nature Genet. 1996 **12** 17-23]

hKCNQ2: Human KCNQ2 [*Biervert et al.*, Science 1998 279, 403-406]

hKCNQ3: Human KCNQ3 [*Schroeder et al.*, Nature 1998 396, 687-690]

hKCNQ4: Human KCNQ4; A protein of the invention

- : No amino acid in this position.

* : Indicates positions which have a single, fully conserved residue (Conserved regions).

Biological Activity

The polynucleotide of the invention encodes a potassium channel subunit, which has been termed KNCQ4. In the cochlea, it is differentially expressed in sensory outer hair cells. A mutation in this gene in a pedigree with autosomal dominant hearing loss changes a residue in the KCNQ4 pore region. It abolishes the outwardly rectifying potassium currents of wild-type KCNQ4 on which it exerts a strong dominant negative effect.

Ion channels are excellent targets for drugs. KCNQ4, or heteromeric channels containing the KCNQ4 subunit, may be a particularly interesting target for the treatment of tinnitus and the prevention or treatment of progressive hearing loss.

KCNQ Channels in Genetic Disease

It is remarkable that mutations in every known *KCNQ* gene lead to human disease: Mutations in *KCNQ1* (KvLQT1) cause the autosomal dominant long QT syndrome (LQTS), and, when present on both alleles, the Jervell and Lange-Nielsen

(JLN) syndrome whose symptoms include deafness in addition to cardiac arrhythmias. Mutations in either *KCNQ2* or *KCNQ3*, which form heteromers that probably represent the M-channel, cause benign familial neonatal convulsions (BFNC). The present invention adds *KCNQ4* and the associated autosomal dominant deafness to that list.

After KCNQ1, KCNQ4 is now the second KCNQ channel whose loss of function leads to deafness.

Mutant DNA Sequences / Variant Proteins

Therefore, in a preferred embodiment of the invention, mutated polynucleotides may be employed in the screening for drugs that affect diseases associated with such mutations in the *KCNQ4* gene.

In the context of this invention, the term "mutated polynucleotide" means a polynucleotide (or DNA sequence) having a nucleotide sequence that differs from the sequence presented as SEQ ID NO: 1 at one or more nucleotide positions.

The mutated polynucleotide may in particular be a polynucleotide of the invention having a nucleotide sequence as in SEQ ID NO: 1, which sequence, however, differs from SEQ ID NO: 1 so as to effect the expression of a variant polypeptide. The mutated polynucleotide may be a polynucleotide of the invention having a nucleotide sequence encoding a potassium channel having an amino acid sequence that has been changed at one or more positions. The mutated polynucleotide may in particular be a polynucleotide of the invention having a nucleotide sequence encoding a potassium channel having an amino acid sequence that has been changed at one or more positions located in the conserved regions, as defined by Table 1, above.

In a more specific embodiment the polynucleotide of the invention has the polynucleotide sequence giving rise to the G285S mutation as indicated in SEQ ID NO: 1, i.e. the DNA sequence that at position 935-937 holds the codon AGC rather than the codon GGC stated in SEQ ID NO: 1.

In the context of this invention, the term "variant polypeptide" means a polypeptide (or protein) having an amino acid sequence that differs from the sequence presented as SEQ ID NO: 2 at one or more amino acid positions.

In a most specific embodiment, variants of this protein are also contemplated according to the invention, including splice variants, isoforms,

homologues from other species, and polymorphisms, and mutations including the variant KCNQ4/G285S (i.e. KCNQ4/G333S according to the KCNQ1 numbering).

Heteromers Formed by KCNQ Subunits

The KCNQ channels described so far function physiologically as heteromers. KCNQ1 associates with KCNE1 (formerly known as minK), and KCNQ2 and KCNQ3 form heteromeric channels that underlie the M-current, an important determinant of neuronal excitability that is regulated by several neurotransmitters.

Like other KCNQ channel subunits, KCNQ4 may interact with other subunits, e.g. KCNE1 or other KCNQ channel subunits, and in particular with KCNQ3. Currents from homomeric KCNQ3 are very small and often cannot be distinguished from *Xenopus* oocyte background currents. Co-expression of KCNQ3 with KCNQ4 markedly increased current amplitudes. Significantly, heteromeric KCNQ3/KCNQ4 channels activated faster than homomeric KCNQ4 channels, the voltage-dependence was shifted to more negative potentials, and currents displayed a different drug sensitivity.

Genetically Manipulated Cells

In a third aspect the invention provides a cell genetically manipulated by the incorporation of the heterologous polynucleotide of the invention. The cell of the invention may in particular be genetically manipulated to transiently or stably express, over-express or co-express a KCNQ4 channel subunit as defined above.

In a preferred embodiment, the cell of the invention is an eukaryotic cell, in particular a mammalian cell, an oocyte, or a yeast cell.

In a more preferred embodiment, the of the invention is a human embryonic kidney (HEK) cell, a HEK 293 cell, a BHK21 cell, a Chinese hamster ovary (CHO) cell, a *Xenopus laevis* oocyte (XLO) cell, or any other cell line able to express KCNQ potassium channels.

KCNQ4 Active Chemical Compounds

In another aspect the invention relates to chemical compounds capable of binding to, and showing activity at potassium channels containing one or more KCNQ4

subunits. In the context of this invention such compounds are termed KCNQ4 active compounds.

The KCNQ4 active compounds of the invention have therapeutic potential, and may be used for the manufacture of pharmaceutical compositions.

The KCNQ4 active compounds of the invention may in particular be used in diagnosis, treatment, prevention or alleviation of diseases related to tinnitus, loss of hearing, in particular progressive hearing loss, neonatal deafness, and presbyacusis (deafness of the elderly); and diseases or adverse conditions of the CNS, including affective disorders, Alzheimer's disease, anxiety, ataxia, CNS damage caused by trauma, stroke or neurodegenerative illness, cognitive deficits, compulsive behaviour, dementia, depression, Huntington's disease, mania, memory impairment, memory disorders, memory dysfunction, motion disorders, motor disorders, neurodegenerative diseases, Parkinson's disease and Parkinson-like motor disorders, phobias, Pick's disease, psychosis, schizophrenia, spinal cord damage, stroke, and tremor.

Currently two compound have been identified. As a preferred embodiment the invention therefore provides 1,3-dihydro-1-phenyl-3,3-bis(4-pyridylmethyl)-2H-indol-2-one (Linopirdine) and 10,10-bis(4-pyridinyl-methyl)-9(10H)-antracenone (XE991) for use in the manufacture of a pharmaceutical composition for the diagnosis, treatment, prevention or alleviation of the above diseases.

Screening of Drugs

In a further aspect the invention provides methods for screening for KCNQ4 active compounds, i.e. chemical compounds capable of binding to, and showing activity at potassium channels containing one or more KCNQ4 subunits. The activity determined may be inhibitory activity, stimulating activity, or other modulatory activity.

Such chemical compounds can be identified by one of, or both methods described below.

Binding Studies

Binding studies are usually carried out by subjecting the target to binding with a labelled, selective agonist (binding agent), to form a labelled complex, followed by determination of the degree of displacement caused by the test compound upon addition to the complex.

In a specific aspect the invention provides a method of screening a chemical compound for capability of binding to a potassium channel comprising at least one KCNQ4 channel subunit, which method comprises the steps of (i) subjecting a KCNQ4 channel subunit containing cell to the action of a KCNQ4 binding agent to form a complex with the KCNQ4 channel subunit containing cell; (ii) subjecting the complex of step (i) to the action of the chemical compound to be tested; and (iii) detecting the displacement of the KCNQ4 binding agent from the complex with the KCNQ4 channel subunit containing cell.

The KCNQ4 channel subunit containing cell preferably is a cell of the invention as described above.

The KCNQ4 binding agent preferably is a radioactively labelled 1,3-dihydro-1-phenyl-3,3-bis(4-pyridylmethyl)-2H-indol-2-one (Linopirdine); or 10,10-bis(4-pyridinyl-methyl)-9(10H)-antracenone.

In a even more preferred embodiment, the biding agent is labelled with ^3H, and the displacement of the KCNQ4 binding agent from the complex with the KCNQ4 channel subunit containing cell is detected by measuring the amount of radioactivity by conventional liquid scintillation counting.

Activity Studies

The KCNQ4 channel agonists may affect the potassium channel in various ways. The agonist may in particular show inhibitory activity, stimulating activity, or other modulatory activity.

In a specific aspect the invention provides a method for determining the activity at potassium channels containing one or more KCNQ4 subunits. According to this method a KCNQ4 channel subunit containing cell is subjecting to the action of the chemical compound to be tested, and the activity is detected by way of monitoring the membrane potential, the current, the potassium flux, or the secondary calcium influx of the KCNQ4 channel subunit containing cell, preferably a genetically manipulated as described above.

The membrane potential and the current may be monitored by electrophysiologic methods, including patch clamp techniques, such as current clamp technology and two-electrode voltage clamp technology, or by spectroscopic methods, such as fluorescence methods.

In a preferred embodiment, monitoring of the membrane potential of the KCNQ4 channel subunit containing cell is performed by patch clamp techniques.

In another preferred embodiment, monitoring of the membrane potential of the KCNQ4 channel subunit containing cell is performed by spectroscopic methods, e.g. using fluorescence methods. In a more specific embodiment, the KCNQ4 channel subunit containing cell is mixed with a membrane potential indicating agent, that allow for a determination of changes in the membrane potential of the cell, caused by the addition of the test compound. The membrane potential indicating agent may in particular be a fluorescent indicator, preferably $DIBAC_4(3)$, DiOC5(3), and DiOC2(3).

In yet a preferred embodiment, monitoring of the membrane potential of the KCNQ4 channel subunit containing cell is performed by spectroscopic methods, e.g. using a FLIPR assay (Fluorescence Image Plate Reader; available from Molecular Devices).

Screening of Genetic Material

In a further aspect the invention relates to the use of a polynucleotide sequence of the invention for the screening of genetic materials. By this method, individuals bearing a gene identical or homologous to a polynucleotide of the invention may be identified.

In the screening method of the invention, a polynucleotide of the invention, or any fragment or subsequence hereof, is employed. For the identification of individuals bearing mutated genes preferably the mutated forms of the polynucleotide represented by SEQ ID NO: 1 are employed, in particular a polynucleotide sequence holding the mutation giving rise to the KCNQ4/G285S variant.

In the screening method of the invention only short sequences needs to be employed depending on the actual method used. For SSCA, several hundreds of base pairs may be needed, for oligonucleotide or PCR hybridisation only of from about 10 to about 50 basepairs may be needed.

In a more specific embodiment, the primer sequences used in Example 2 below for the amplification of the single KCNQ4 exons may be used for the screening of mutations.

The screening may be accomplished by conventional methods, including hybridisation, SSCA analysis, and array technology (DNA chip technology). The

hybridisation protocol described above represents a suitable protocol for use in a screening method of the invention.

Transgenic Animals

Transgenic animal models provide the means, *in vivo*, to screen for therapeutic compounds. The establishment of transgenic animals may in particular be helpful for the screening of drugs to fully elucidate the pathophysiology of *KCNQ4*/DFNA2 deafness. These animals may also be valuable as a model for the frequent condition of presbyacusis that also develops slowly over decades. Since KCNQ4 is expressed also in brain, they may also be helpful in screening for drugs effective in CNS disorders, e.g. epilepsy.

By transgene is meant any piece of polynucleotide which is inserted by artifice into a cell, and thus becomes part of the genome of the organism that develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e. foreign) to the transgenic organism, or it may represent a gene homologous to an endogenous gene of the organism.

By a transgenic animal is meant any organism holding a cell which includes a polynucleotide sequence which is inserted into that cell by artifice, and which cell becomes part of the transgenic organism which develops from that cell. Such a transgene may be partly or entirely heterologous to the transgenic animal. Although transgenic mice represent a preferred embodiment of the invention, other transgenic mammals including, but not limited to transgenic rodents (e.g. hamsters, guinea pigs, rabbits and rats), and transgenic pigs, cattle, sheep and goats may be created by standard techniques and are included in the invention.

Preferably, the transgene is inserted by artifice into the nuclear genome.

<u>Knock-out and Knock-in Animals</u>

The transgenic knock-out animal models may be developed by homologous recombination of embryonic stem cells with constructs containing genomic sequence from the *KCNQ4* gene, that lead to a loss of function of the gene after insertion into the endogenous gene.

By knock-out mutation is meant an alteration in the polynucleotide sequence that reduces the biological activity of the polypeptide normally encoded

therefrom. In order to create a true knock-out model, the biological activity of the expressed polypeptide should be reduced by at least 80% relative to the unmutated gene. The mutation may in particular be a substitution, an insertion, a deletion, a frameshift mutation, or a mis-sense mutation. Preferably the mutation is a substitution, an insertion or a deletion.

To further assess the role of KCNQ4 at an organism level, the generation of an animal, preferably a mouse, lacking the intact *KCNQ4* gene, or bearing a mutated *KCNQ4* gene, is desired.

A replacement-type targeting vector, which may be used to create a knock-out model, may be constructed using an isogenic genomic clone, e.g. from a mouse strain such as 129/Sv (Stratagene Inc., La Jolla, CA). The targeting vector may be introduced into a suitably-derived line of embryonic stem (ES) cells by electroporation to generate ES cell lines that carry a profoundly truncated form of the *KCNQ4* gene. The targeted cell lines may then be injected into a mouse blastula stage embryo to generate chimeric founder mice. Heterozygous offspring may be interbred to homozygosity.

As the slowly progressive hearing loss observed in DFNA2 may require the expression from one allele of a dominant negative mutant, it may also be desired to create a knock-in animal in which the wild-type *KCNQ4* gene is replaced by this mutated gene.

Animal models for overexpression may be generated by integrating one or more polynucleotide sequence of the invention into the genome according to standard techniques.

The procedures disclosed herein involving the molecular manipulation of nucleic acids are known to those skilled in the art, and are described by e.g. *Fredrick MA et al.* [*Fredrick MA et al.*: Short Protocols in Molecular Biology; John Wiley and Sons, 1995] and *Sambrook et al.* [*Sambrook et al.*: Molecular Cloning: A Laboratory Manual; 2. Ed., Cold Spring Harbor Lab.; Cold Spring Harbor, NY 1989], and in *Alexandra LJ* (Ed.): Gene Targeting: A practical approach; Oxford University Press (Oxford, New York, Tokyo), 1993.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated by reference to the accompanying drawing, in which:

Fig. 1 shows the electrophysiological properties of KCNQ4 currents: **(A)** Two-electrode voltage-clamp current traces from a *Xenopus* oocyte injected with KCNQ4 cRNA. Starting from a holding potential of –60 mV cells were clamped for 4 s to voltages between -80...+60 mV in +10 mV steps, followed by a constant test pulse to –30 mV. **(B)** Current traces showing the inactivation behaviour of KCNQ4 at different voltages. After an activating voltage pulse at +40 mV of 3.5 s duration the cell was clamped to voltages between +40...-120 mV in –10 mV steps. (**C**) Apparent open-probability (p_{open}) as a function of voltage determined from tail current analysis of currents as in (A). Half-maximal p_{open} is archived at (–10.0±1.2) mV, and the apparent gating charge is 1.4±0.1, as obtained from a fit of a Boltzmann-function to the data (n=14 from 2 oocyte batches, ±S.E.M.). (**D**) Shift of the reversal potential with the extracellular K^+-concentration with ND98 as reference solution. Total monovalent cation concentration was 100 mM and the stated K^+-concentration was obtained by mixing solutions ND100 and KD100. The reversal potential shift of 46.7±0.9 mV per decade indicates a channel selective for K^+ (n=18 from 3 oocyte batches, ±S.E.M.). Substitution of external K^+ with other cations yielded the following permeability ratios: P_K/P_{Na} = 52.3±4.4, P_K/P_{Cs} = 7.8±0.7, and P_K/P_{Rb} = 0.94±0.03 (permeability sequence: $Rb^+ \sim K^+ > Cs^+ >> Na^+$, n=15 from 3 oocyte batches, ±S.E.M.). (**E**) Current traces of WT KCNQ4 (thick solid line), a 1:1 coinjection of WT KCNQ4 and $KCNQ4_{G285S}$ mutant (thin solid line) and $KCNQ4_{G285S}$ mutant (dotted line). $KCNQ4_{G285S}$ currents were indistinguishable from water-injected control oocytes. From a holding potential at –60 mV the cells were voltage-clamped for 6 s at +40 mV, followed by a –30 mV step. **(F)** Mean currents, measured after clamping oocytes for 4 s at +40 mV, averaged from several experiments as in (E) (n=20...35, 4 oocyte batches, ±S.E.M.); and

Fig. 2 shows the co-expression of KCNQ4 with KCNQ1 (**A**), KCNQ2 (**B**), and KCNQ3 (**C**) and derived dominant negative mutants ($KCNQ1_{G219S}$, $KCNQ2_{G279S}$, $KCNQ3_{G318S}$) (n=10...31, 3 oocyte batches, ±S.E.). (**D**) Representative currents from experiments as in (C) showing altered activation kinetics for the coinjection of KCNQ4 with KCNQ3 or $KCNQ3_{G318S}$, respectively. From a holding potential at –60 mV the

voltage was clamped for 4 s at +40 mV, followed by a step to –30 mV. Time constants and amplitudes obtained from two-exponential fits were: KCNQ4: t_1=360 ms, A_1=-4.9 μA, t_2=1700 ms, A_2=-0.34 μA; KCNQ3+KCNQ4: t_1=120 ms, A_1=-6.3 μA, t_2=560ms, A_2=-1.3 μA. (**E**) Apparent p_{open} as a function of voltage for currents from oocytes coinjected with KCNQ4 and KCNQ3 cRNA, determined from tail current analysis (squares, thick solid curve). Half-maximal p_{open} is achieved at $V_{0.5}$ = (–19.1 ± 2.0) mV, and the apparent gating charge is 1.5±0.2 (n=23 from 3 oocyte batches, ±S.E.M.), as obtained from a fit of a Boltzmann-function to the data. The p_{open} curve for KCNQ4 is also shown for reference (circles, thin solid curve). (**F**) Current traces recorded from an oocyte coinjected with KCNQ3 and KCNQ4 cRNA with typical M-current voltage-protocol. Starting from a holding potential at –30 mV the cell was progressively hyperpolarized for 1 s to voltages between –30 and –90 mV in –10 mV steps. (**G**) Differential effects of 200 μM Linopirdine on KCNQ4 (n=10, ±S.E.M.) and KCNQ3+KCNQ4 (n=6, ±S.E.M.). Currents were measured at +40 mV and % current remaining with Linopirdine is shown. Upon addition of Linopirdine steady-state inhibition was reached after ~1 min for KCNQ4 currents, and after ~3 min for KCNQ3+KCNQ4 currents.

EXAMPLES

The invention is further illustrated with reference to the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

Example 1

Cloning and Characterisation of KCNQ4 cDNA

Using a KCNQ3 potassium channel partial cDNA as a probe, a human retina cDNA λ phage library (Clontech, #HL1132a) was screened, and a ≈1 kb cDNA encoding a protein fragment homologous to KCNQ potassium channels was isolated. It was distinct from the known members KCNQ1 (KvLQT1), KCNQ2 and KCNQ3. We named the novel gene *KCNQ4*. Overlapping cDNA's containing the entire open reading frame were obtained by rescreening the cDNA library and by extending the 5' end in RACE (rapid amplification of cDNA ends) experiments using a Marathon kit (Clontech) with human skeletal muscle cDNA. A complete cDNA was assembled and

cloned into the oocyte expression vector PTLN [*Lorenz C, Pusch M & Jentsch T J*: Heteromultimeric CLC chloride channels with novel properties; Proc. Natl. Acad. Sci. USA 1996 **93** 13362-13366].

The cDNA encodes a polypeptide of 695 amino acids with a predicted mass of 77 kDa (SEQ ID NO: 2). Its overall amino-acid identity to KCNQ1, KCNQ2, and KCNQ3 is 38%, 44%, and 37%, respectively. Together with these proteins it forms a distinct branch of the superfamily of voltage-gated potassium channels. As a typical member of this gene family, KCNQ4 has 6 predicted transmembrane domains and a P-loop between transmembrane domains S5 and S6. In potassium channels, which are tetramers of identical or homologous subunits, four of these highly conserved P-loops combine to form the ion-selective pore. As other KCNQ channels, KCNQ4 has a long predicted cytoplasmic carboxyterminus that accounts for about half of the protein. A conserved region present in the carboxytermini of KCNQ1, -2, and -3 is also present in KCNQ4 (roughly represented by exon 12).

The sequence of KCNQ4 predicts several potential sites for phosphorylation by protein kinase C. In contrast to KCNQ1 and KCNQ2, however, it lacks an aminoterminal consensus site for cAMP-dependent phosphorylation.

A human multiple tissue Northern blot (Clontech, #7760-1) was probed with a 749 bp EcoRI/PmlI cDNA fragment of KCNQ4. The fragment was labelled with ^{32}P using the Rediprime labelling kit (Amersham). Hybridisation was performed in ExpressHyb solution according to the instructions of the manufacturer (Clontech). The filter was then exposed to Kodak BioMax film for 4 days.

Northern analysis of *KCNQ4* expression in human tissues revealed faint bands of ≈5 kb in heart, brain and skeletal muscle. In some tissues, there was also a larger band. Upon longer exposure, weaker ≈5 kb bands were also detected in other tissues including kidney and pancreas.

Example 2

Genomic structure and chromosomal mapping to the DFNA2 locus

A PAC was isolated that contains the entire *KCNQ4* coding region. The genomic structure of the *KCNQ4* gene was established (SEQ ID NO: 1).

The genomic structure was established by a PCR approach from genomic DNA. Individual *KCNQ4* exons and adjacent short intronic sequences were amplified

by standard PCR techniques from human genomic DNA using intronic oligonucleotide primers.

For amplification, the following intronic primer pairs were used (all primers in 5´→3´ direction; in brackets the size of the PCR product):

1a: catgcgtctctgagcgccccgagc	1r: aggccaggcttgcgcggggaaacg	(544)
2a: cagcacagagctgtaactccagg	2r: aagctgctctctgagccatgg	(500)
3a: gctgggtccgcgctgtgacc	3r: ggtctccagggtcagagtcg	(292)
4a: tccgggtccgtgcgcggggta	4r: gagacagcccctctgacctcg	(328)
5a: atccctttcccgtgtggaagc	5r: agtcacgatgggcagacctcg	(286)
6a: cctcatgatcaggctcctacc	6r: atgtgtgacaggggtgagc	(270)
7a: aaggatggggacacccttgc	7r: acacagggttgacacacc	(244)
8a: gctctgggtaacccacaactg	8r: gctcccctgggagccatcacc	(316)
9a: tgagctcaggagctctgtgc	9r: acccacgaagtggctgaaggc	(346)
10a: gtcctaagtcagctttgtcc	10r: cctcagccggccctcgatcg	(347)
11a: cactctactggtggtttggc	11r: ctcctgacctcaagtgatcc	(281)
12a: gatagcaaagagatggagagg	12r: aactcagctgcagcagtgagc	(328)
13a: gtgccttctccttcatcaggc	13r: aacgcatcctccccatgtca	(297)
14a: tttgtgcttcccagataagc	14r: cgtgagggagtgagttcaagtacg	(445)

Sequences of exons and adjacent introns are deposited in GenBank (Accession Numbers AF105203-AF105216).

To screen unlinked pedigrees with autosomal dominant deafness we amplified only exons 4 through 7 that code for the pore and adjacent transmembrane domains as these may have the highest likelihood to harbour mutations. After amplification and agarose gel purification, PCR products were directly sequenced using the amplification primers and an ABI377 automated DNA sequencer.

The highly conserved transmembrane block S1-S6 was found to be encoded by 6 exons (exons 2 to 7) having the same limits as in KCNQ2 and KCNQ3. In KCNQ1 an additional intron interrupts the sequence encoding domain S4. The exon-intron structures of KCNQ genes diverge most in the poorly conserved carboxy-termini of these proteins.

Using hybridisation to human chromosomes, *KCNQ4* was mapped to 1p34.

A PAC containing the coding sequence of *KCNQ4* was isolated using intronic *KCNQ4* oligonucleotide primers and PCR. It was used to localise *KCNQ4* to 1p34 using FISH (Genome Systems). *KCNQ4* was then mapped on the Whitehead Contig WC1.10 using several of the intronic primers given above and published STS markers by PCR amplification from individual YAC clones.

Several diseases have been mapped to this region. This includes DFNA2, a locus for dominant progressive hearing loss. Due to the critical role of K^+ homeostasis in auditory mechanotransduction, we considered *KCNQ4* as an excellent candidate gene for DFNA2. The DFNA2 locus has been mapped between markers D1S255 and D1S193. We therefore refined the localisation of *KCNQ4* in comparison to published physical and genetic maps using a YAC (yeast artificial chromosome) contig of this region. *KCNQ4* was present on CEPH YAC clone 914c3, a result which places this gene within the DFNA2 region.

Example 3

Expression of KCNQ genes in the inner ear

The expression of *KCNQ4*, as well as of other KCNQ genes, was studied by semiquantitative RT-PCR on mouse cochlear RNA.

<u>RT-PCR analysis of mouse KCNQ mRNA expression</u>

Approximately 2 µg of mouse total brain RNA and mouse cochlear and vestibular RNA were reverse transcribed using the SuperScript™ II (Gibco BRL) reverse transcriptase.

1 µl (resp. 1 µl of a 1:10 dilution) of cDNA was amplified for 30 cycles (96°C for 30 sec, 61°C for 30 sec, and 68°C for 45 sec) using a 2400 Thermocycler System (Perkin Elmer). Each 50 µl reaction contained 2.5 U polymerase (Expand™ Long Template PCR System, Boehringer Mannheim) and 5% DMSO.

KCNQ1 primers were based on the mouse cDNA sequence (GenBank Accession # U70068):

MK1a 5´-aaggctggatcagtccattgg-3´; and

MK1r 5´-aggtgggcaggctgttgctgg-3´ (280 bp).

As no mouse KCNQ2 sequence was available, we chose sequences conserved between human (Y15065) and rat (AF087453) KCNQ2:

MK2a 5´-gccacggcacctcccccgtgg-3´; and

MK2r 5´-ccctctgcaatgtagggcctgac-3´ (331 bp).

KCNQ3 primers were derived from a mouse EST (AA386747):

MK3a 5´-ccaaggaatgaaccatatgtagcc-3´; and

MK3r 5´-cagaagagtcaagatgggcaggac-3´ (461 bp).

Mouse *KCNQ4* primers were:

MK4a 5´-agtacctgatggagcgccctctcg-3´; and

MK4r 5´-tcatccaccgtaagctcacactgg-3´ (366 bp).

Amplification products were verified by direct sequencing.

These results were compared with those obtained with vestibular and brain RNA.

KCNQ1, *KCNQ3*, and *KCNQ4* messages can be detected in the cochlea, and additional PCR cycles revealed a weak *KCNQ2* expression as well. At this high amplification, KCNQ1 was also detected in brain. *KCNQ1* and *KCNQ4* appear to have the highest cochlear expression. *KCNQ1* expression is higher in the cochlea than in brain (which was negative by Northern analysis). The reverse is true for *KCNQ2* and *KCNQ3*, both of which are broadly expressed in brain. *KCNQ4* expression is significant in both of these tissues.

In situ hybridisation of mouse cochlea

In situ hybridisation's were performed on cochlea sections from mice at postnatal day P12 with a *KCNQ4* antisense probe.

A mouse *KCNQ4* cDNA corresponding to bp 618 to 1602 of the human *KCNQ4* ORF was cloned into pBluescript. Sense and antisense probes were transcribed using T3 and T7 RNA polymerases after appropriate linearization. After DNase digestion, the probes were ethanol precipitated twice with 0.4 M LiCl. They were labelled with digoxigenin-11-UTP as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993).

Mouse inner ears were fixed for 1 hour at 4 °C in 4 % paraformaldehyde in PBS. After three rinses in PBS, they were immersed in 20 % sucrose overnight at 4°C. Cryostat sections (10-14 μm) were postfixed and rinsed in PBS. Following pre-hybridisation at room temperature for at least 3 hours, they were hybridised overnight at 58 °C in a humid chamber. Sections were then washed and incubated with sheep

antidigoxigenin antibody coupled to alkaline phosphatase. Staining by NBT/BCIP (Boehringer Mannheim) was done for 2 hours at 37°C and overnight at RT. Sections were then mounted in Aquatex (Merck, USA).

Sensory outer hair cells were strongly labelled. By contrast, the inner hair cells appeared negative. The stria vascularis, the site of *KCNQ1* expression, was negative as well. Control hybridisation with a *KCNQ4* sense probe revealed that the staining of outer hair cells was specific.

<u>Autosomal Dominant Deafness</u>

These results indicated that *KCNQ4* was an excellent candidate gene for autosomal dominant deafness. As we did not have access to the published pedigrees that were linked to the DFNA2 locus, we screened 45 families with autosomal dominant deafness without previous linkage analysis. In most of these families, the hearing loss had been diagnosed before adulthood, i.e. before the age of onset reported for most of the DFNA forms, including DFNA2.

Mutation screening was limited to exons 4 to 7 that encode the pore region and adjacent transmembrane domains. A *KCNQ4* mutation was found in a French family with profound hearing loss. Its clinical features include progressive hearing loss that is more prominent with higher frequencies, tinnitus in one patient, and no indication for vestibular defects nor gross morphological changes in the inner ear. A mis-sense mutation (cf. SEQ ID NO: 1; The mutation G935A at the nucleotide level giving rise to the variant G285S at the amino acid level) was present in exon 6 in a heterozygous state. Using an AluI restriction site (AGCT) introduced by this mutation, it was shown that it co-segregated with all affected members in the pedigree. This mutation was not found on 150 control Caucasian chromosomes.

The G285S mutation affects the first glycine in the GYG signature sequence of potassium channel pores. This glycine is highly conserved across different classes of potassium channels in all species. The crystal structure of the *Streptomyces lividans* potassium channel reveals that these three amino acids line the narrowest part of the ion-conductive pore. Mutations in these amino-acids disrupt the selectivity filter and in most cases lead to a loss of channel function. Interestingly, an identical change in amino acids at the equivalent position was found in the *KCNQ1* gene of a patient with the dominant long QT syndrome. It disrupted channel activity and exerted a dominant

negative effect on co-expressed WT KCNQ1 channels. These mutations also have dominant negative effects when inserted into *KCNQ2* and *KCNQ3*. This is strong evidence that the progressive hearing loss in this family is due to the KCNQ4/G285S mutation.

Example 4

Functional expression of KCNQ4 potassium channel subunits

KCNQ4 was expressed in *Xenopus* oocytes and its activity was investigated by two-electrode voltage clamping.

After linearization of the *KCNQ4*-containing PTLN vector with HpaI, capped cRNA was transcribed *in vitro* using the mMessage mMachine cRNA synthesis kit (Ambion). Usually 5 – 15 ng of cRNA were injected into *Xenopus* oocytes previously isolated by manual defolliculation and short collagenase treatment. In co-expression experiments cRNAs were injected at a 1:1 ratio. Oocytes were kept at 17°C in modified Barth's solution (90 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 10 mM HEPES, 100 U penicillin–100 µg streptomycin/ml, pH 7.6).

Standard two-electrode voltage-clamp measurements were performed at room temperature 2-4 days after injection using a Turbotec 05 amplifier (npi instruments, Tamm, Germany) and pClamp 5.5 software (Axon Instruments). Currents were usually recorded in ND98 solution (see Table 2). Solutions for Na^+ / K^+ replacement experiments were prepared from an appropriate mixture of solution KD100 (100 mM KCl) and ND100 (100 mM NaCl) to yield the stated concentrations of Na^+ and K^+. Linopirdine (RBI, Natick, MA) was prepared as a 100 mM stock solution in DMSO and added to a final concentration of 200 µM to ND98.

Table 2

Solution contents (concentrations in mM)

ND98	ND 100	KD100	Rb100	Cs100
98 NaCl 2 KCl 0.2 $CaCl_2$ 2.8 $MgCl_2$	100 NaCl 0.2 $CaCl_2$ 2.8 $MgCl_2$	100 KCl 0.2 $CaCl_2$ 2.8 $MgCl_2$	100 RbCl 0.2 $CaCl_2$ 2.8 $MgCl_2$	100 CsCl 0.2 $CaCl_2$ 2.8 $MgCl_2$
5 mM HEPES, pH 7.4				

Reversal potentials were determined from tail currents after a 2 s depolarising pulse to +60 mV and corrected for liquid junction potentials that were determined experimentally. The permeability ratios were calculated according to P_K / P_X = exp ($-F \cdot V_{rev}$ / $R \cdot T$).

To determine the voltage dependence of apparent open probability, oocytes were clamped for 4 s to values between –80 mV to +50 mV in 10 mV steps, followed by a constant –30 mV test pulse. Tail currents extrapolated to t=0 were obtained from a monoexponential fit, normalised to the value at 0 mV and used for the analysis of apparent p_{open}. Data analysis used PClamp6 and Microcal Origin 5.0.

Similar to KCNQ1, KCNQ2 and KCNQ3, also KCNQ4 yielded currents that activated upon depolarisation (Fig. 1A). Compared to those other KCNQ channels, however, current activation was slower and occurred with a time constant in the order of 600 ms at + 40mV (KCNQ2/KCNQ3 channels have a corresponding time constant of ≈300 ms). This time constant was very sensitive to changes in temperature. Deactivation of currents at physiological resting potentials (≈-70mV) was considerably faster (Fig. 1B). Similar to KCNQ2, macroscopic currents often showed some inward rectification at positive potentials. When oocytes were depolarised to +60 mV for 10 sec or more, an apparent slow inactivation of currents was observed that resembled the one described for KCNQ3. Currents began to activate at about -40 mV, with half-maximal activation at -10 mV (Fig. 2C). Ion substitution experiments showed that the channel is highly selective for potassium (Fig. 1D). It has a $K^+ \approx Rb^+ > Cs^+ > Na^+$

permeability sequence. KCNQ4 currents were inhibited by more than 80% by 5 mM Ba^{++}.

We next examined the effect of the G285S mutation found in the affected family (Figs. 1E and 1F). The mutant channel did not yield any detectable currents in the *Xenopus* oocyte expression system. $KCNQ4_{G285S}$ was then injected at a 1:1 ratio with WT KCNQ4 to mimic the situation in a heterozygous DFNA2 patient. This reduced currents by about 90%, indicating a strong dominant negative effect of the mutant. The degree of current reduction is compatible with the notion that the incorporation of one mutant subunit suffices to abolish the function of the tetrameric channel complex. The channels present in co-injected oocytes still showed a strong preference of potassium over sodium or calcium. This implies that the deafness is due to a quantitative loss of KCNQ4 potassium currents rather than to an influx of sodium or calcium.

KCNQ1 assembles with minK (IsK) to form channels that yield larger currents and activate much slower. We therefore tested by co-expression whether minK affects KCNQ4 as well. At concentrations (1ng minK cRNA per oocyte) leading to drastic changes in KCNQ1 currents in parallel experiments, there was no significant change in KCNQ4 currents.

Different KCNQ subunits can form heteromeric channels. Co-expression of KCNQ2 with KCNQ3, but not with KCNQ1, gave currents that were about tenfold larger than those from homomeric channels. Since also KCNQ1 and KCNQ3 (and to a lesser degree also KCNQ2) are expressed in the cochlea, we investigated whether these proteins interact functionally. Oocytes co-injected (at the same total cRNA concentration) with KCNQ1 and KCNQ4 cRNAs yielded currents that seemed not different from a linear superposition of currents from the respective homomeric channels (Fig. 2A), and the same was true for oocytes co-expressing KCNQ2 and KCNQ4 (Fig. 2B). In addition, a dominant negative KCNQ1 mutant did not suppress KCNQ4 currents (Fig. 2A), and the same was true for the equivalent KCNQ2 mutant (Fig. 2B).

By contrast, co-expression of KCNQ3 with KCNQ4 yielded currents that were significantly larger than could be explained by a superposition of currents from the respective homomeric channels (Figs. 2C and 2D). Further, KCNQ4 currents were markedly suppressed by co-expressing a dominant negative KCNQ3 mutant (Fig. 2C).

(Fig. 2D), and there was a ≈10 mV shift of the open probability towards negative voltages (Fig. 2E). Compared to KCNQ2/KCNQ3 channels, which may underlie the M-current, KCNQ3/KCNQ4 heteromers open at slightly more positive voltages. To compare KCNQ3/KCNQ4 channels to M-channels, we used the typical voltage-protocol employed for these channels and found currents superficially resembling M-currents (Fig. 2F). Linopirdine, a potent and rather specific inhibitor for M-currents, nearly completely inhibits KCNQ2/KCNQ3 channels at a concentration of 200 μM. This concentration of Linopirdine inhibited KCNQ4 by about 30%, while a significantly larger inhibition (≈75%) was observed with KCNQ3/KCNQ4 co-expression (Fig. 2G).

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2335 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(vii) IMMEDIATE SOURCE:

(B) CLONE: KCNQ4

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:83..2170

(ix) FEATURE:

(A) NAME/KEY: mutation

(B) LOCATION:replace(935, "")

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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AGCCATGCGT CTCTGAGCGC CCCGAGCGCG CCCCCGCCCC GGACCGTGCC CGGGCCCCGG      60

CGCCCCCAGC CCGGCGCCGC CC ATG GCC GAG GCC CCC CCG CGC CGC CTC GGC      112
                         Met Ala Glu Ala Pro Pro Arg Arg Leu Gly
                          1               5                  10

CTG GGT CCC CCG CCC GGG GAC GCC CCC CGC GCG GAG CTA GTG GCG CTC       160
Leu Gly Pro Pro Pro Gly Asp Ala Pro Arg Ala Glu Leu Val Ala Leu
                15                  20                  25

ACG GCC GTG CAG AGC GAA CAG GGC GAG GCG GGC GGG GGC GGC TCC CCG       208
Thr Ala Val Gln Ser Glu Gln Gly Glu Ala Gly Gly Gly Gly Ser Pro
            30                  35                  40

CGC CGC CTC GGC CTC CTG GGC AGC CCC CTG CCG CCG GGC GCG CCC CTC       256
Arg Arg Leu Gly Leu Leu Gly Ser Pro Leu Pro Pro Gly Ala Pro Leu
        45                  50                  55

CCT GGG CCG GGC TCC GGC TCG GGC TCC GCC TGC GGC CAG CGC TCC TCG       304
Pro Gly Pro Gly Ser Gly Ser Gly Ser Ala Cys Gly Gln Arg Ser Ser
    60                  65                  70
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GCC GCG CAC AAG CGC TAC CGC CGC CTG CAG AAC TGG GTC TAC AAC GTG        352
Ala Ala His Lys Arg Tyr Arg Arg Leu Gln Asn Trp Val Tyr Asn Val
 75                  80                  85                  90

CTG GAG CGG CCC CGC GGC TGG GCC TTC GTC TAC CAC GTC TTC ATA TTT        400
Leu Glu Arg Pro Arg Gly Trp Ala Phe Val Tyr His Val Phe Ile Phe
                 95                 100                 105

TTG CTG GTC TTC AGC TGC CTG GTG CTG TCT GTG CTG TCC ACT ATC CAG        448
Leu Leu Val Phe Ser Cys Leu Val Leu Ser Val Leu Ser Thr Ile Gln
            110                 115                 120

GAG CAC CAG GAA CTT GCC AAC GAG TGT CTC CTC ATC TTG GAA TTC GTG        496
Glu His Gln Glu Leu Ala Asn Glu Cys Leu Leu Ile Leu Glu Phe Val
        125                 130                 135

ATG ATC GTG GTT TTC GGC TTG GAG TAC ATC GTC CGG GTC TGG TCC GCC        544
Met Ile Val Val Phe Gly Leu Glu Tyr Ile Val Arg Val Trp Ser Ala
    140                 145                 150

GGA TGC TGC TGC CGC TAC CGA GGA TGG CAG GGT CGC TTC CGC TTT GCC        592
Gly Cys Cys Cys Arg Tyr Arg Gly Trp Gln Gly Arg Phe Arg Phe Ala
155                 160                 165                 170

AGA AAG CCC TTC TGT GTC ATC GAC TTC ATC GTG TTC GTG GCC TCG GTG        640
Arg Lys Pro Phe Cys Val Ile Asp Phe Ile Val Phe Val Ala Ser Val
                175                 180                 185

GCC GTC ATC GCC GCG GGT ACC CAG GGC AAC ATC TTC GCC ACG TCC GCG        688
Ala Val Ile Ala Ala Gly Thr Gln Gly Asn Ile Phe Ala Thr Ser Ala
            190                 195                 200

CTG CGC AGC ATG CGC TTC CTG CAG ATC CTG CGC ATG GTG CGC ATG GAC        736
Leu Arg Ser Met Arg Phe Leu Gln Ile Leu Arg Met Val Arg Met Asp
        205                 210                 215

CGC CGC GGC GGC ACC TGG AAG CTG CTG GGC TCA GTG GTC TAC GCG CAT        784
Arg Arg Gly Gly Thr Trp Lys Leu Leu Gly Ser Val Val Tyr Ala His
    220                 225                 230

AGC AAG GAG CTG ATC ACC GCC TGG TAC ATC GGG TTC CTG GTG CTC ATC        832
Ser Lys Glu Leu Ile Thr Ala Trp Tyr Ile Gly Phe Leu Val Leu Ile
235                 240                 245                 250
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TTC GCC TCC TTC CTG GTC TAC CTG GCC GAG AAG GAC GCC AAC TCC GAC      880
Phe Ala Ser Phe Leu Val Tyr Leu Ala Glu Lys Asp Ala Asn Ser Asp
                255                 260                 265

TTC TCC TCC TAC GCC GAC TCG CTC TGG TGG GGG ACG ATT ACA TTG ACA      928
Phe Ser Ser Tyr Ala Asp Ser Leu Trp Trp Gly Thr Ile Thr Leu Thr
            270                 275                 280

ACC ATC GGC TAT GGT GAC AAG ACA CCG CAC ACA TGG CTG GGC AGG GTC      976
Thr Ile Gly Tyr Gly Asp Lys Thr Pro His Thr Trp Leu Gly Arg Val
        285                 290                 295

CTG GCT GCT GGC TTC GCC TTA CTG GGC ATC TCT TTC TTT GCC CTG CCT     1024
Leu Ala Ala Gly Phe Ala Leu Leu Gly Ile Ser Phe Phe Ala Leu Pro
    300                 305                 310

GCC GGC ATC CTA GGC TCC GGC TTT GCC CTG AAG GTC CAG GAG CAG CAC     1072
Ala Gly Ile Leu Gly Ser Gly Phe Ala Leu Lys Val Gln Glu Gln His
315                 320                 325                 330

CGG CAG AAG CAC TTC GAG AAG CGG AGG ATG CCG GCA GCC AAC CTC ATC     1120
Arg Gln Lys His Phe Glu Lys Arg Arg Met Pro Ala Ala Asn Leu Ile
                335                 340                 345

CAG GCT GCC TGG CGC CTG TAC TCC ACC GAT ATG AGC CGG GCC TAC CTG     1168
Gln Ala Ala Trp Arg Leu Tyr Ser Thr Asp Met Ser Arg Ala Tyr Leu
            350                 355                 360

ACA GCC ACC TGG TAC TAC TAT GAC AGT ATC CTC CCA TCC TTC AGA GAG     1216
Thr Ala Thr Trp Tyr Tyr Tyr Asp Ser Ile Leu Pro Ser Phe Arg Glu
        365                 370                 375

CTG GCC CTC TTG TTT GAG CAC GTG CAA CGG GCC CGC AAT GGG GGC CTA     1264
Leu Ala Leu Leu Phe Glu His Val Gln Arg Ala Arg Asn Gly Gly Leu
    380                 385                 390

CGG CCC CTG GAG GTG CGG CGG GCG CCG GTA CCC GAC GGA GCA CCC TCC     1312
Arg Pro Leu Glu Val Arg Arg Ala Pro Val Pro Asp Gly Ala Pro Ser
395                 400                 405                 410

CGT TAC CCG CCC GTT GCC ACC TGC CAC CGG CCG GGC AGC ACC TCC TTC     1360
Arg Tyr Pro Pro Val Ala Thr Cys His Arg Pro Gly Ser Thr Ser Phe
                415                 420                 425
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TGC CCT GGG GAA AGC AGC CGG ATG GGC ATC AAA GAC CGC ATC CGC ATG      1408
Cys Pro Gly Glu Ser Ser Arg Met Gly Ile Lys Asp Arg Ile Arg Met
            430                 435                 440

GGC AGC TCC CAG CGG CGG ACG GGT CCT TCC AAG CAG CAG CTG GCA CCT      1456
Gly Ser Ser Gln Arg Arg Thr Gly Pro Ser Lys Gln Gln Leu Ala Pro
        445                 450                 455

CCA ACA ATG CCC ACC TCC CCA AGC AGC GAG CAG GTG GGT GAG GCC ACC      1504
Pro Thr Met Pro Thr Ser Pro Ser Ser Glu Gln Val Gly Glu Ala Thr
    460                 465                 470

AGC CCC ACC AAG GTG CAA AAG AGC TGG AGC TTC AAT GAC CGC ACC CGC      1552
Ser Pro Thr Lys Val Gln Lys Ser Trp Ser Phe Asn Asp Arg Thr Arg
475                 480                 485                 490

TTC CGG GCA TCT CTG AGA CTC AAA CCC CGC ACC TCT GCT GAG GAT GCC      1600
Phe Arg Ala Ser Leu Arg Leu Lys Pro Arg Thr Ser Ala Glu Asp Ala
                495                 500                 505

CCC TCA GAG GAA GTA GCA GAG GAG AAG AGC TAC CAG TGT GAG CTC ACG      1648
Pro Ser Glu Glu Val Ala Glu Glu Lys Ser Tyr Gln Cys Glu Leu Thr
            510                 515                 520

GTG GAC GAC ATC ATG CCT GCT GTG AAG ACA GTC ATC CGC TCC ATC AGG      1696
Val Asp Asp Ile Met Pro Ala Val Lys Thr Val Ile Arg Ser Ile Arg
        525                 530                 535

ATT CTC AAG TTC CTG GTG GCC AAA AGG AAA TTC AAG GAG ACA CTG CGA      1744
Ile Leu Lys Phe Leu Val Ala Lys Arg Lys Phe Lys Glu Thr Leu Arg
    540                 545                 550

CCG TAC GAC GTG AAG GAC GTC ATT GAG CAG TAC TCA GCA GGC CAC CTG      1792
Pro Tyr Asp Val Lys Asp Val Ile Glu Gln Tyr Ser Ala Gly His Leu
555                 560                 565                 570

GAC ATG CTG GGC CGG ATC AAG AGC CTG CAA ACT CGG GTG GAC CAA ATT      1840
Asp Met Leu Gly Arg Ile Lys Ser Leu Gln Thr Arg Val Asp Gln Ile
                575                 580                 585

GTG GGT CGG GGG CCC GGG GAC AGG AAG GCC CGG GAG AAG GGC GAC AAG      1888
Val Gly Arg Gly Pro Gly Asp Arg Lys Ala Arg Glu Lys Gly Asp Lys
            590                 595                 600
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GGG CCC TCC GAC GCG GAG GTG GTG GAT GAA ATC AGC ATG ATG GGA CGC      1936
Gly Pro Ser Asp Ala Glu Val Val Asp Glu Ile Ser Met Met Gly Arg
        605                 610                 615

GTG GTC AAG GTG GAG AAG CAG GTG CAG TCC ATC GAG CAC AAG CTG GAC      1984
Val Val Lys Val Glu Lys Gln Val Gln Ser Ile Glu His Lys Leu Asp
    620                 625                 630

CTG CTG TTG GGC TTC TAT TCG CGC TGC CTG CGC TCT GGC ACC TCG GCC      2032
Leu Leu Leu Gly Phe Tyr Ser Arg Cys Leu Arg Ser Gly Thr Ser Ala
635                 640                 645                 650

AGC CTG GGC GCC GTG CAA GTG CCG CTG TTC GAC CCC GAC ATC ACC TCC      2080
Ser Leu Gly Ala Val Gln Val Pro Leu Phe Asp Pro Asp Ile Thr Ser
                655                 660                 665

GAC TAC CAC AGC CCT GTG GAC CAC GAG GAC ATC TCC GTC TCC GCA CAG      2128
Asp Tyr His Ser Pro Val Asp His Glu Asp Ile Ser Val Ser Ala Gln
            670                 675                 680

ACG CTC AGC ATC TCC CGC TCG GTC AGC ACC AAC ATG GAC TGA              2170
Thr Leu Ser Ile Ser Arg Ser Val Ser Thr Asn Met Asp  *
        685                 690                 695

GGGACTTCTC AGAGGCAGGG CAGCACACGG CCAGCCCCGC GGCCTGGCGC TCCGACTGCC    2230

CTCTGAGGCC TCCGGACTCC TCTCGTACTT GAACTCACTC CCTCACGGGG AGAGAGACCA    2290

CACGCAGTAT TGAGCTGCCT GAGTGGGCGT GGTACCTGCT GTGGG                    2335
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 696 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Met Ala Glu Ala Pro Pro Arg Arg Leu Gly Leu Gly Pro Pro Pro Gly
 1               5                  10                  15

Asp Ala Pro Arg Ala Glu Leu Val Ala Leu Thr Ala Val Gln Ser Glu
            20                  25                  30
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Gln Gly Glu Ala Gly Gly Gly Gly Ser Pro Arg Arg Leu Gly Leu Leu
35 40 45

Gly Ser Pro Leu Pro Pro Gly Ala Pro Leu Pro Gly Pro Gly Ser Gly
50 55 60

Ser Gly Ser Ala Cys Gly Gln Arg Ser Ser Ala Ala His Lys Arg Tyr
65 70 75 80

Arg Arg Leu Gln Asn Trp Val Tyr Asn Val Leu Glu Arg Pro Arg Gly
85 90 95

Trp Ala Phe Val Tyr His Val Phe Ile Phe Leu Leu Val Phe Ser Cys
100 105 110

Leu Val Leu Ser Val Leu Ser Thr Ile Gln Glu His Gln Glu Leu Ala
115 120 125

Asn Glu Cys Leu Leu Ile Leu Glu Phe Val Met Ile Val Val Phe Gly
130 135 140

Leu Glu Tyr Ile Val Arg Val Trp Ser Ala Gly Cys Cys Cys Arg Tyr
145 150 155 160

Arg Gly Trp Gln Gly Arg Phe Arg Phe Ala Arg Lys Pro Phe Cys Val
165 170 175

Ile Asp Phe Ile Val Phe Val Ala Ser Val Ala Val Ile Ala Ala Gly
180 185 190

Thr Gln Gly Asn Ile Phe Ala Thr Ser Ala Leu Arg Ser Met Arg Phe
195 200 205

Leu Gln Ile Leu Arg Met Val Arg Met Asp Arg Arg Gly Gly Thr Trp
210 215 220

Lys Leu Leu Gly Ser Val Val Tyr Ala His Ser Lys Glu Leu Ile Thr
225 230 235 240

Ala Trp Tyr Ile Gly Phe Leu Val Leu Ile Phe Ala Ser Phe Leu Val
245 250 255

Tyr Leu Ala Glu Lys Asp Ala Asn Ser Asp Phe Ser Ser Tyr Ala Asp
260 265 270

Ser Leu Trp Trp Gly Thr Ile Thr Leu Thr Thr Ile Gly Tyr Gly Asp
275 280 285

Lys Thr Pro His Thr Trp Leu Gly Arg Val Leu Ala Ala Gly Phe Ala
290 295 300

Leu Leu Gly Ile Ser Phe Phe Ala Leu Pro Ala Gly Ile Leu Gly Ser
305 310 315 320

Gly Phe Ala Leu Lys Val Gln Glu Gln His Arg Gln Lys His Phe Glu
325 330 335

Lys Arg Arg Met Pro Ala Ala Asn Leu Ile Gln Ala Ala Trp Arg Leu
340 345 350

Tyr Ser Thr Asp Met Ser Arg Ala Tyr Leu Thr Ala Thr Trp Tyr Tyr
355 360 365

Tyr Asp Ser Ile Leu Pro Ser Phe Arg Glu Leu Ala Leu Leu Phe Glu
370 375 380

His Val Gln Arg Ala Arg Asn Gly Gly Leu Arg Pro Leu Glu Val Arg
385 390 395 400

Arg Ala Pro Val Pro Asp Gly Ala Pro Ser Arg Tyr Pro Pro Val Ala
405 410 415

Thr Cys His Arg Pro Gly Ser Thr Ser Phe Cys Pro Gly Glu Ser Ser
420 425 430

Arg Met Gly Ile Lys Asp Arg Ile Arg Met Gly Ser Ser Gln Arg Arg
435 440 445

Thr Gly Pro Ser Lys Gln Gln Leu Ala Pro Pro Thr Met Pro Thr Ser
450 455 460

Pro Ser Ser Glu Gln Val Gly Glu Ala Thr Ser Pro Thr Lys Val Gln
465 470 475 480

Lys Ser Trp Ser Phe Asn Asp Arg Thr Arg Phe Arg Ala Ser Leu Arg
485 490 495

Leu Lys Pro Arg Thr Ser Ala Glu Asp Ala Pro Ser Glu Glu Val Ala
500 505 510

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Glu Glu Lys Ser Tyr Gln Cys Glu Leu Thr Val Asp Asp Ile Met Pro
        515                 520                 525

Ala Val Lys Thr Val Ile Arg Ser Ile Arg Ile Leu Lys Phe Leu Val
    530                 535                 540

Ala Lys Arg Lys Phe Lys Glu Thr Leu Arg Pro Tyr Asp Val Lys Asp
545                 550                 555                 560

Val Ile Glu Gln Tyr Ser Ala Gly His Leu Asp Met Leu Gly Arg Ile
                565                 570                 575

Lys Ser Leu Gln Thr Arg Val Asp Gln Ile Val Gly Arg Gly Pro Gly
            580                 585                 590

Asp Arg Lys Ala Arg Glu Lys Gly Asp Lys Gly Pro Ser Asp Ala Glu
        595                 600                 605

Val Val Asp Glu Ile Ser Met Met Gly Arg Val Val Lys Val Glu Lys
    610                 615                 620

Gln Val Gln Ser Ile Glu His Lys Leu Asp Leu Leu Leu Gly Phe Tyr
625                 630                 635                 640

Ser Arg Cys Leu Arg Ser Gly Thr Ser Ala Ser Leu Gly Ala Val Gln
                645                 650                 655

Val Pro Leu Phe Asp Pro Asp Ile Thr Ser Asp Tyr His Ser Pro Val
            660                 665                 670

Asp His Glu Asp Ile Ser Val Ser Ala Gln Thr Leu Ser Ile Ser Arg
        675                 680                 685

Ser Val Ser Thr Asn Met Asp  *
    690                 695
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CLAIMS

1. An isolated polynucleotide having a nucleic acid sequence which is capable of hybridising under high stringency conditions with the polynucleotide sequence presented as SEQ ID NO: 1, its complementary strand, or a sub-sequence thereof.

2. The isolated polynucleotide according to claim 1, being at least 50% homologous, preferably more than 70%, more preferred more than 80%, even more preferred more than 90%, most preferred more than 95%, homologous to the polynucleotide sequence presented as SEQ ID NO: 1.

3. The isolated polynucleotide according to either of claims 1-2 being a cloned polynucleotide.

4. The isolated polynucleotide according to claim 3, in which the polynucleotide is cloned from, or produced on the basis of a cDNA library.

5. The isolated polynucleotide according to any of claims 1-4, having the polynucleotide sequence presented as SEQ ID NO: 1.

6. The isolated polynucleotide according to any of claims 1-4, having the polynucleotide sequence presented as SEQ ID NO: 1, including the mutation G935A.

7. The isolated polynucleotide according to any of claims 1-6, encoding a potassium channel, or a potassium channel subunit.

8. The isolated polynucleotide according to claim 7, encoding the KCNQ4 potassium channel subunit having the amino acid sequence represented by SEQ ID NO: 2.

9. The isolated polynucleotide according to claim 7, encoding a KCNQ4 variant, which variant has an amino acid sequence that has been changed by deletion of

an amino acid residue, by insertion of an additional amino acid residue, or by substitution of an amino acid residue at one or more positions.

10. The isolated polynucleotide according to claim 9, which variant has an amino acid sequence that has been changed at one or more positions located in the conserved regions, as defined by Table 1.

11. The isolated polynucleotide according to claim 9, encoding the variant KCNQ4/G285S (i.e. KCNQ4/G333S according to the KCNQ1 numbering).

12. A recombinantly produced polypeptide encoded by the polynucleotide according to claims 1-11.

13. The polypeptide according to claim 12, being a KCNQ4 potassium channel subunit having the amino acid sequence presented as SEQ ID No. 2.

14. The polypeptide according to claim 12, being a KCNQ4 variant, which variant has an amino acid sequence that has been changed by deletion of an amino acid residue, by insertion of an additional amino acid residue, or by substitution of an amino acid residue at one or more positions.

15. The polypeptide according to claim 14, which variant has an amino acid sequence that has been changed at one or more positions located in the conserved regions, as defined by Table 1.

16. The polypeptide according to claim 14, being the variant KCNQ4/G285S (i.e. KCNQ4/G333S according to the KCNQ1 numbering).

17. A cell genetically manipulated by the incorporation of a heterologous polynucleotide according to any of claims 1-11.

18. The cell according to claim 17, genetically manipulated by the incorporation of a KCNQ4 channel subunit having the amino acid sequence presented as SEQ ID NO: 2.

19. The cell according to claim 17, genetically manipulated by the incorporation of a KCNQ4 variant, which variant has an amino acid sequence that has been changed by deletion of an amino acid residue, by insertion of an additional amino acid residue, or by substitution of an amino acid residue at one or more positions.

20. The cell according to claim 19, which variant has an amino acid sequence that has been changed at one or more positions located in the conserved regions, as defined by Table 1.

21. The cell according to claim 19, genetically manipulated by the incorporation of the variant KCNQ4/G285S (i.e. KCNQ4/G333S according to the KCNQ1 numbering).

22. The cell according to any of claims 17-21, genetically manipulated to co-express one or more KCNQ channel subunits.

23. The cell according to claim 22, genetically manipulated to co-express KCNQ4 and KCNQ1 channel subunits; KCNQ4 and KCNQ2 channel subunits; KCNQ4 and KCNQ3 channel subunits; KCNQ4 and KCNQ1 and KCNQ2 channel subunits; KCNQ4 and KCNQ1 and KCNQ3 channel subunits; KCNQ4 and KCNQ2 and KCNQ3 channel subunits; or KCNQ4 and KCNQ1 and KCNQ2 and KCNQ3 channel subunits.

24. The cell according to claim 22, genetically manipulated to co-express KCNQ3 and KCNQ4 channel subunits.

25. The cell according to any of claims 17-24, being an eukaryotic cell, in particular a mammalian cell, an oocyte, or a yeast cell.

26. The cell according to any claim 25, being a human embryonic kidney (HEK) cell, a HEK 293 cell, a BHK21 cell, a Chinese hamster ovary (CHO) cell, a *Xenopus laevis* oocyte (XLO) cell, or any other cell line able to express KCNQ potassium channels.

27. A method of screening a chemical compound for capability of binding to a potassium channel comprising at least one KCNQ4 channel subunit, which method comprises the steps of
    (i) subjecting a KCNQ4 channel subunit containing cell to the action of a KCNQ4 binding agent to form a complex with the KCNQ4 channel subunit containing cell;
    (ii) subjecting the complex of step (i) to the action of the chemical compound to be tested; and
    (iii) detecting the displacement of the KCNQ4 binding agent from the complex with the KCNQ4 channel subunit containing cell.

28. The method of claim 27, wherein the KCNQ4 channel subunit containing cell is a cell according to any of claims 17-26.

29. The method of either of claims 27-28, in which the KCNQ4 binding agent is
    (i) radioactively labelled 1,3-dihydro-1-phenyl-3,3-bis(4-pyridylmethyl)-2H-indol-2-one (Linopirdine); or
    (ii) radioactively labelled 10,10-bis(4-pyridinyl-methyl)-9(10H)-antracenone;

30. The method of claim 29, which compounds have been marked with ^3H.

31. The method of either of claims 29-30, wherein the displacement of the KCNQ4 binding agent from the complex with the KCNQ4 channel subunit containing cell is detected by measuring the amount of radioactivity by conventional liquid scintillation counting.

32. A method of screening a chemical compound for activity on a potassium channel comprising at least one KCNQ4 channel subunit, which method comprises the steps of
    (i) subjecting a KCNQ4 channel subunit containing cell to the action of the chemical compound; and
    (ii) monitoring the membrane potential, the current, the potassium flux, or the secondary calcium influx of the KCNQ4 channel subunit containing cell.

33. The method of claim 32, wherein the KCNQ4 channel subunit containing cell is a cell according to any of claims 17-26.

34. The method of either of claims 32-33, wherein monitoring of the membrane potential of the KCNQ4 channel subunit containing cell is performed by patch clamp techniques.

35. The method of either of claims 32-33, wherein monitoring of the membrane potential of the KCNQ4 channel subunit containing cell is performed using fluorescence methods.

36. A chemical compound identified by the method of claims 27-31, and/or by claims 32-35.

37. Use of the chemical compound according to claim 36 for diagnosis, treatment, prevention or alleviation of diseases related to tinnitus, loss of hearing, in particular progressive hearing loss, neonatal deafness, and presbyacusis (deafness of the elderly); and diseases or adverse conditions of the CNS, including affective disorders, Alzheimer's disease, anxiety, ataxia, CNS damage caused by trauma, stroke or neurodegenerative illness, cognitive deficits, compulsive behaviour, dementia, depression, Huntington's disease, mania, memory impairment, memory disorders, memory dysfunction, motion disorders, motor disorders, neurodegenerative diseases, Parkinson's disease and

Parkinson-like motor disorders, phobias, Pick's disease, psychosis, schizophrenia, spinal cord damage, stroke, and tremor.

38. The use according to claim 37, wherein the chemical compound is 1,3-dihydro-1-phenyl-3,3-bis(4-pyridylmethyl)-2H-indol-2-one (Linopirdine); or 10,10-bis(4-pyridinyl-methyl)-9(10H)-antracenone.

39. Use of a polynucleotide sequence according to any of claims 1-11, for the screening of genetic materials for individuals having this mutations.

40. A transgenic animal comprising a knock-out mutation of the endogenous *KCNQ4* gene, a mutated *KCNQ4* gene, or genetically manipulated in order to over-express the *KCNQ4* gene or to over-express mutated *KCNQ4* gene.

41. The transgenic animal according to claim 40, being a knock-out animal in which the gene is totally deleted in a homozygous state.

42. The transgenic animal according to claim 40, comprising a mutated *KCNQ4* gene.

43. The transgenic animal according to any of claims 40-42, being a transgenic rodent, in particular a hamster, a guinea pig, a rabbit, or a rat, a transgenic pig, a transgenic cattle, a transgenic sheep, or a transgenic goat.

44. Use of the transgenic animal according to any of claims 40-43 for the *in vivo* screening of therapeutic compounds.

45. The use according to claim 44, for the screening of drugs affecting diseases or conditions associated with hearing loss or tinnitus.

TITLE: **NOVEL POTASSIUM CHANNELS AND GENES ENCODING THESE POTASSIUM CHANNELS**

ABSTRACT

This invention relates to novel potassium channels and genes encoding these channels. More specifically the invention provides isolated polynucleotides encoding the KCNQ4 potassium channel, cells transformed with these polynucleotides, transgenic animals comprising genetic mutations, and the use of the transformed cells and the transgenic animals for the *in vitro* and *in vivo* screening of drugs affecting KCNQ4 containing potassium channels.

Modtaget PD

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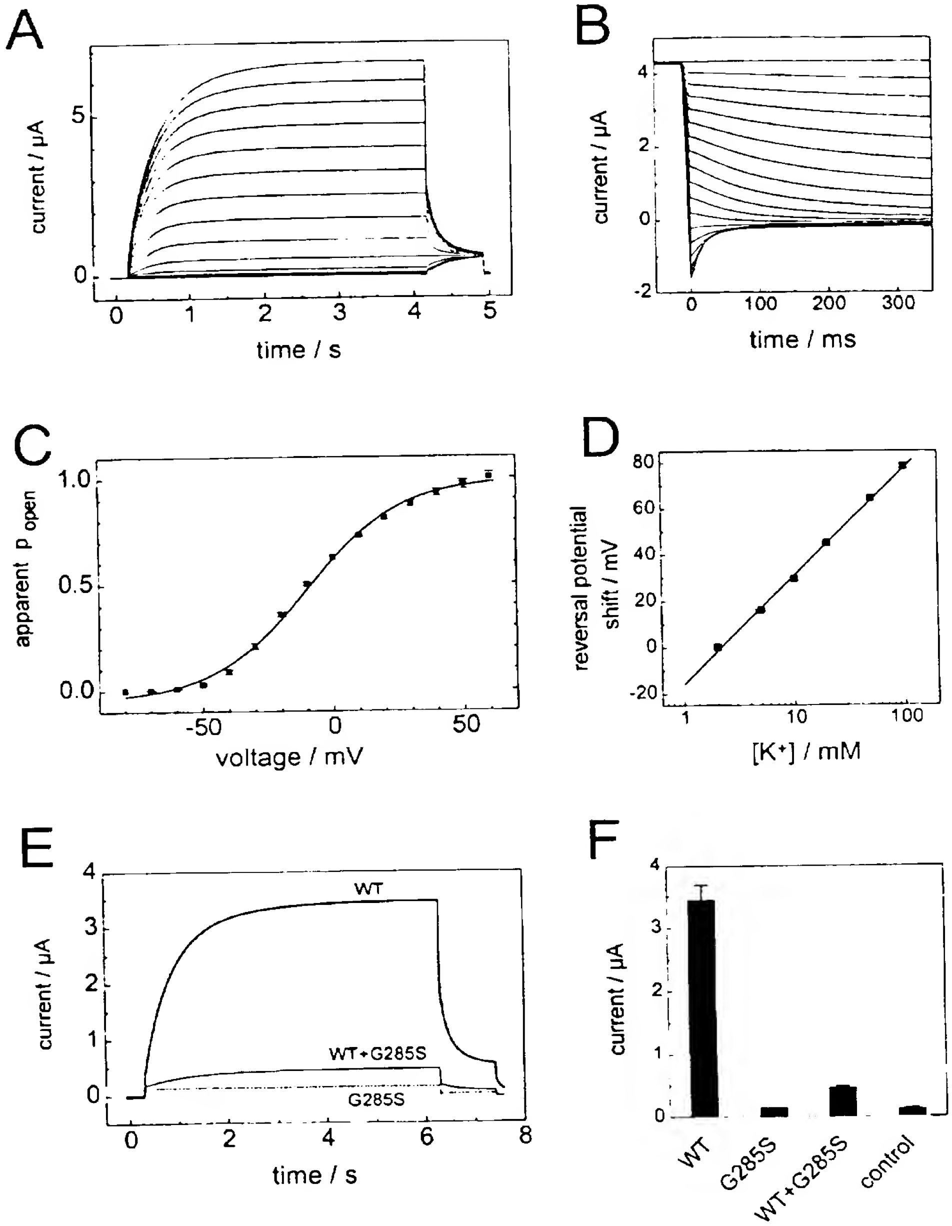

Fig. 1

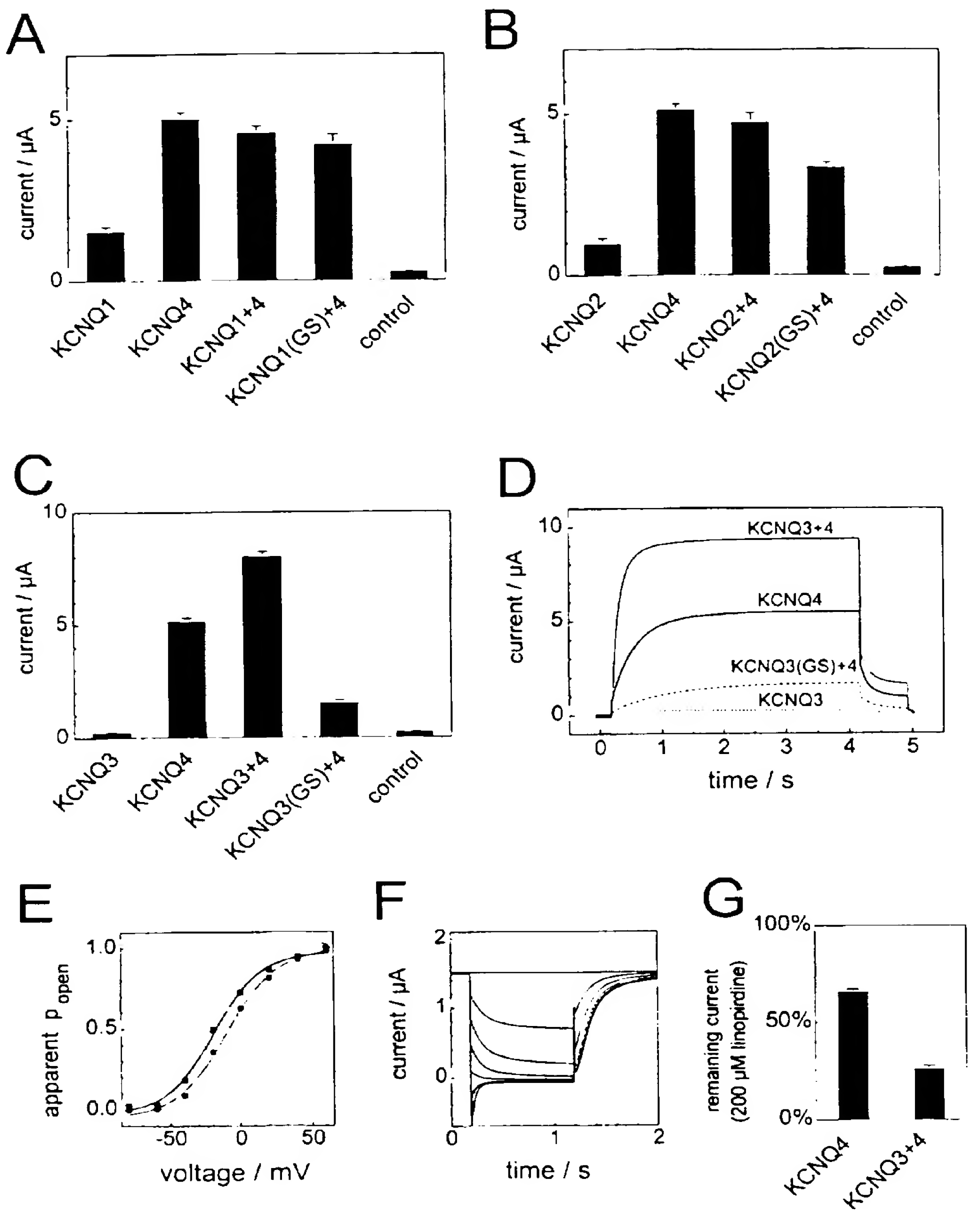
A
current / µA
0
5
KCNQ1
KCNQ4
KCNQ1+4
KCNQ1(GS)+4
control
B
current / µA
0
5
KCNQ2
KCNQ4
KCNQ2+4
KCNQ2(GS)+4
control
C
current / µA
0
5
10
KCNQ3
KCNQ4
KCNQ3+4
KCNQ3(GS)+4
control
D
current / µA
0
5
10
KCNQ3+4
KCNQ4
KCNQ3(GS)+4
KCNQ3
0
1
2
3
4
5
time / s
E
apparent p open
0.0
0.5
1.0
-50
0
50
voltage / mV
F
current / µA
0
1
2
0
1
2
time / s
G
remaining current
(200 µM linopirdine)
0%
50%
100%
KCNQ4
KCNQ3+4

Fig. 2